PBR
Pharmaceutical & Biomedical Research

July 2022. Volume 8. Number 3

Original Article

Comparing the Effects of Consuming Propolis and Chicory for Eight Weeks and Resistance Training on Histopathological and Morphometric Changes of Uterine Tissue in Rats Treated With Testosterone Enanthate

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Article info:
Received: 08 Nov 2021
Accepted: 18 Dec 2021

Keywords:
Resistance training, Testosterone enanthate, Propolis, Chicory, Uterus histology

ABSTRACT

Background: The illegal use of anabolic and androgenic steroids is a public health problem and their side effects in women are different from the side effects observed in men.

Objectives: This study aims to compare the effects of consuming propolis and chicory for 8 weeks and resistance training on histopathological and morphometric changes of the uterine tissue in rats treated with testosterone enanthate.

Methods: In this experimental study, 40 female Wistar rats were randomly divided into 5 groups (n=8): control (placebo), sham (resistance training), resistance training plus testosterone enanthate (20 mg/kg body weight), resistance training plus testosterone enanthate+chicory (6 g/kg body weight), and resistance training plus testosterone enanthate plus propolis (400 mg/kg body weight). The resistance training protocol was performed 5 sessions per week in 4 rounds with an intensity of 40% to 160% of the body weight of the mice for 8 weeks. After weighing and dissection, the uterine tissue was examined histologically.

Results: The thickness of the endometrial layer in the sham, testosterone, and propolis groups showed a significant decrease compared to the control group ($P<0.05$). There was a significant decrease in the thickness of the functional layer in the sham, testosterone, chicory, and propolis groups compared to the control group ($P<0.05$). The thickness of the basal layer showed a significant decrease in the testosterone and propolis groups compared to the control group ($P<0.05$). Glands in the testosterone, chicory, and propolis groups showed a significant decrease compared to the control group ($P<0.05$). The thickness of the myometrial layer showed a significant decrease in the testosterone group compared to the control, sham, chicory, and propolis groups ($P<0.05$).

Conclusion: The results showed that high-intensity resistance training combined with the use of testosterone enanthate causes histopathological changes in the uterus of female rats. Meanwhile, the use of propolis and chicory can improve the effects of testosterone enanthate as a treatment option.

Citation Movahed S, Akbarpour Beni M, Cheraghi E. Comparing the Effects of Consuming Propolis and Chicory for Eight Weeks and Resistance Training on Histopathological and Morphometric Changes of Uterine Tissue in Rats Treated With Testosterone Enanthate. Pharmaceutical and Biomedical Research. 2022; 8(3):189-198.

http://dx.doi.org/10

Introduction

Resistance training has become an essential part of health, fitness, and exercise programs through different types of resistance training methods [1]. Today, most athletes, especially bodybuilders, seek to increase muscle mass through resistance training with nutrition and supplements. Accordingly, in the last decade, the use of anabolic-androgenic steroids (AAS) as drugs to increase muscle function and volume has substantially increased among beginner and professional athletes and bodybuilders [2]. AAS are synthetic derivatives of the male hormone testosterone [3]. Taking AAS and resistance training increase various factors, such as physical function, lean mass, muscle size, strength, and collagen synthesis [4].

With reported use of 6.4% in men and 1.6% in women, testosterone enanthate and testosterone propionate comprise 87% of performance-enhancing drugs among athletes [3]. Although the use of such substances is higher among men than women, the increasing trend of use among women has raised many concerns about gender, hormonal, and fertility changes [5]. Therefore, the illegal use of anabolic and androgenic steroids is a public health problem; meanwhile, the medical side effects are completely different in women who take steroids from the side effects observed in men. Numerous side effects, such as hoarse sound, enlargement of the clitoris, changes in menstruation, hirsutism, and male pattern baldness are common clinical features of hyperandrogenism in women; many of them may not be transient in women consumers compared to men [6, 7]. Anabolic steroids can dramatically affect a woman's reproductive cycle [8]. In menarche, delayed menopause, dysmenorrhea, oligomenorrhea, secondary amenorrhea, and infertility are the changes most commonly associated with steroid abuse. A study has shown that the administration of nandrolone, an AAS in female rats alters their uterine morphology and reduces their reproductive capacity [9]. The misuse of these drugs can eventually lead to harmful histopathological changes in the uterus and ovaries and the modification of the hormonal array of women's biology, such as the reduction in the luteinizing hormone [10].

Despite the destructive effects of steroid use, which is increasing every day, it is necessary to pay attention to treatment and elimination of the side effects of steroid use. Therefore, to solve these problems, experts use chemical or herbal scavengers. Despite the beneficial effects, chemical scavengers have side effects [11]; accordingly, experts and researchers have attended to the use of herbal scavengers. Therefore, it is necessary to identify scavengers that can have the most preventive or deterrent effects with the least side effects, such as propolis and chicory.

Propolis (bee glue) is a dark-colored substance collected by the bee from living plants. It is combined with wax and used in the construction and adaptation of nests [12]. The chemical structures of propolis contain biologically valuable compounds, including anticancer, antimicrobial, antifungal, antiviral, anti-inflammatory, and antioxidant properties [13]. The results show that this substance is a rich source of phytoestrogens and is a treatment option for diseases related to women [14]. Studies regarding the use of propolis to treat uterine fibroids have concluded that propolis is a new treatment for uterine fibroids [15]. Research has also shown that oral administration of propolis induces estrogenic activity in the estrogen receptors-expressing organs in the body and is a promising treatment option for menopausal symptoms [14].

Chicory is a plant of the Asteraceae family and has highly been attended as a source of prebiotic compounds [16]. Chicory root has various health aspects, such as antiviral, anticancer, antibacterial, anti-inflammatory, antifungal, and antioxidant properties [17]. Some studies have also reported its therapeutic effects against steroids in the liver, kidney, and heart diseases [18].

The use of steroid drugs, such as testosterone enanthate has increased among athletes, especially women, in the last decade. Reportedly, steroid use has several side effects in women, therefore, it is necessary to identify scavengers that have the greatest preventive or inhibitory effects along with the least complications.

Considering that until the implementation of this study, no study has reported the effects of chicory and propolis on women's reproductive health, especially histopathological and morphometric changes in the uterine tissue, this study aims to investigate the effects of consuming propolis and chicory along with resistance training for 8 weeks on histopathological and morphometric changes in the uterine tissue in rats that consume testosterone enanthate.

Materials and Methods

Study design

In this experimental study, 40 female Wistar rats (8 weeks old with an initial weight of 200±12 g), prepared by the Pasteur Institute of Iran, were included in the

study. The animals were kept in groups of 4 with food and water available in special cages (at a temperature of 22°C±2°C, the humidity of 45°C±5°C with a regular cycle of 12 h of light and 12 h of darkness) to adapt to the environment. After 2 weeks of familiarity with the training protocol (Table 1), based on the weight gain, the rats were randomly divided into 5 groups (n=8): control (placebo), sham (resistance training), resistance training plus testosterone enanthate (20 mg/kg body weight), resistance training plus testosterone enanthate plus chicory (6 g/kg body weight), and resistance training plus testosterone enanthate injection plus propolis (400 mg/kg body weight). Sesame oil and distilled water were used as a placebo to dissolve the testosterone enanthate, propolis, and chicory, respectively.

This research has been proved by the Research and Ethics Committee of Qom University (Ethics Code number: IR.QOM.REC.1400.004).

Prescription of drugs

A calibrated insulin syringe was used to administer the drug at an accurate dose and specific time. The deep injection was performed 3 times a week in the serine and back thigh muscles. In the control group, the injection of sesame oil and gavage of distilled water was performed [19]. The appropriate dosage in the experimental groups was obtained based on previous studies, and the testosterone enanthate (LOT=0069) was used at a dose of 20 mg/kg body weight 3 times a week in the form of injection [20]. For the propolis group, propolis was used at a dose of 400 mg/kg body weight in the form of gavage 3 times a week [21]. For the chicory group, chicory was used at a dose of 6 g/kg of body weight in the form of gavage 3 times a week [22]. According to the determination of the period of the estrus cycle in females, dissection was performed in the metestrus stage 24 h following the last session.

Exercise protocol

According to Table 1, the exercise consisted of climbing a 1-meter-high homemade ladder performed by the sham, testosterone, chicory, and propolis groups 5 times a week for 8 weeks [23]. In this exercise, after tying the weight to the rat's tail, they were forced to climb a vertical ladder (90°C). The training was performed 5 days a week (Friday, Saturday, Sunday, Tuesday, and Wednesday) with 2 days of rest in a week (Monday and Thursday) in the morning. The mice were weighed twice a week (Monday and Thursday). The animals became familiar with climbing the ladders during the weeks before the training. Negative amplifiers, such as electric shock, air pressure pump, and so on were never used in the experiment and only hand stimulation and shaking of the animal's tail were used to perform the exercises. The exercises were performed in 4 sets with 6 repetitions and a 90-second rest between the sets along with a 15-second rest between the repetitions. In the first week, the weights attached to the mice were 40% of their body weight, and 20% of their body weight was added every week (the weight-bearing calculations were performed based on twice the weightlifting performed each week), and the workload was gradually increased until 160% of their body weight in the eighth week. In the fifth week, the workload was reduced for a week (to prevent overtraining), in which the workload was 20% less than the previous week. The control group was present at the training site to experience all the existing conditions and to match the stress received from exposure to the examiner.

Evaluating the estrus cycle

Cytology (vaginal swab) was used to evaluate the estrous cycle [24]. The sampling period was considered 7 days. Samples were collected by physiological serum from 9:00 to 11:00 a.m. and were stained and evaluated under a microscope after stabilization using violet crystal dye. The stages of the cycle were as follows: proestrus stage with 2 kinds of cells, including horny and epithelial cells; estrus stage with one kind of cells, including horn cells; metestrus stage with 2 kinds of cells, including horn and leukocytes; and diestrus stage, including one cell population containing leukocytes.

Tissue preparation method

After determining the estrus cycle, the rats were anesthetized and dissected with a combination of ketamin (30-50 mg/kg body weight) and xylazine (3-5 mg/kg body weight) in the metestrus phase. To access the reproductive system, an incision was made with a scalpel on the skin of the abdomen along the middle and pubic bone. After removing the extra appendages and fat, the uterus was removed from the body and weighted with a scale. Then, the left horn of the uterine tissue was sampled. The fragment was placed in 10% formalin. To microscopically study the samples using the technique of preparing microscopic incisions by microtome, pieces with a thickness of 5 to 6 µm were prepared and stained based on the hematoxylin-eosin staining. The endometrial, functional, basal, and myometrial thickness was examined using a lens under a microscope. The number of mucous glands was also counted by a reticular lens in each field of view. For histopathological analysis, using

a Nikon E100 microscope, the thickness of the endometrial layer, functional layer, basal layer, glands, and myometrium was examined [25].

Statistical analysis

The data were reported as Mean±Standard Deviation (SD) and analyzed with a 1-way analysis of variance and the Tukey post hoc test. The analysis of the data was performed by the SPSS software, version 24 (SPSS, Chicago, IL, USA). After ensuring the normal distribution of the data by the Kolmogorov-Smirnov test and the homogeneity of variances based on the Levene test, the statistically significant differences ($P<0.05$) were found.

Results

We found that the body weight of the rats in the sham, testosterone, chicory, and propolis groups had a significant increase compared to the control group. Meanwhile, the uterine weight of the chicory and testosterone groups was significantly increased compared to other groups (Table 2).

The examination of the vaginal cytology showed that the diestrus stage was increased in the testosterone, chicory, and propolis groups, respectively; also, the estrus stage was decreased in these groups. Histological results also demonstrated that in the sham group, the endometrial layer was less thick than in the control group, and the number of glands was reduced. In the testosterone group, the superficial part of the endometrium had lost its tissue structure, and the secretory glands were greatly reduced. In the deep parts, the structural shape was in disarray and its connection with the muscle layer was severed. The covering layer in the endometrium was also changed. The muscles of the myometrial layer were thinner than the control group and the secretory glands showed a significant decrease. In the chicory group, the endometrial layer was improved compared to the testosterone group; however, it still showed degradation and disorganization compared to the control group. In the propolis group, the endometrium layer was improved compared to the testosterone group. In general, the structure of the uterine tissue showed degradation in the propolis group compared to the control group (Figure 1 and 2).

The morphometric results demonstrated that the thickness of the endometrial layer was significantly reduced in the sham group ($P=0.005$), the testosterone group ($P=0.000$), and the propolis group ($P=0.037$) compared to the control group. Also, the testosterone group showed a significant decrease compared to the chicory group ($P=0.014$) (Figure 2A). The thickness of the functional layer was significantly decreased in the sham ($P=0.002$), testosterone ($P=0.000$), chicory ($P=0.004$), and propolis ($P=0.005$) groups compared to the control group. In contrast, the functional layer was not significantly different among sham, testosterone, chicory, and propolis groups (Figure 2B).

The basal layer thickness in the testosterone ($P=0.0001$) and propolis groups ($P=0.001$) showed a significant decrease compared to the control group; meanwhile, the thickness of the basal layer in the testosterone ($P=0.006$), and propolis groups ($P=0.022$) showed a significant decrease compared to the chicory group (Figure 2C). The glands in the testosterone ($P=0.002$), chicory ($P=0.001$), and propolis groups ($P=0.001$) showed a significant decrease compared to the control group. This is while the glands did not show any significant difference between testosterone, chicory, and propolis groups (Figure 2D). The thickness of the myometrial layer in the testosterone group showed a significant decrease compared to the control group ($P=0.000$), sham ($P=0.000$), chicory ($P=0.015$), and propolis ($P=0.0001$) groups. In addition, the thickness of the myometrium layer in the chicory group had a significant decrease compared to the sham ($P=0.048$) and the control groups ($P=0.0001$). The thickness of the myometrial layer was significantly reduced in the propolis group compared to the control group ($P=0.03$) (Figure 2E).

Table 1. Resistance training protocol for female rats

Index \ Week	1	2	3	4	5	6	7	8
Load (g) (%)	40	60	80	100	80	120	140	160
Frequency (repetitions within each set)	6	4	5	6	4	4	5	6
Period (number of set)	1	2	2	2	2	4	4	4
Rest between frequency (second)	15	15	30	30	15	30	30	30
Rest between period (second)	0	60	90	90	60	100	120	120

Table 2. Compassion of body weight and uterine weight in the experimental groups

Index	Experimental Groups					
	Mean±SD					
	Control (Placebo)	Sham (Resistance Training)	Resistance Training +Testosterone	Resistance Training+ Testosterone+Chicory	Resistance Training + Testosterone+Propolis	P
Body weight	211.57±7.3[a]	246.57±23.6[b]	265.85±24.2[b]	240±10.7[b]	245.14±12.8[b]	0.0001
Uterine weight	0.70±0.11[a]	0.55±0.11[b]	0.95±0.1[a]	0.92±0.09[a]	0.97±0.3[a]	0.001

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Notes: The values are shown as Mean±Standard Deviation. In each row, the means with common letter codes do not have a significant difference and the means with different letter codes have a significant difference (1-way analysis of variance, the Tukey test, P≤0.05).

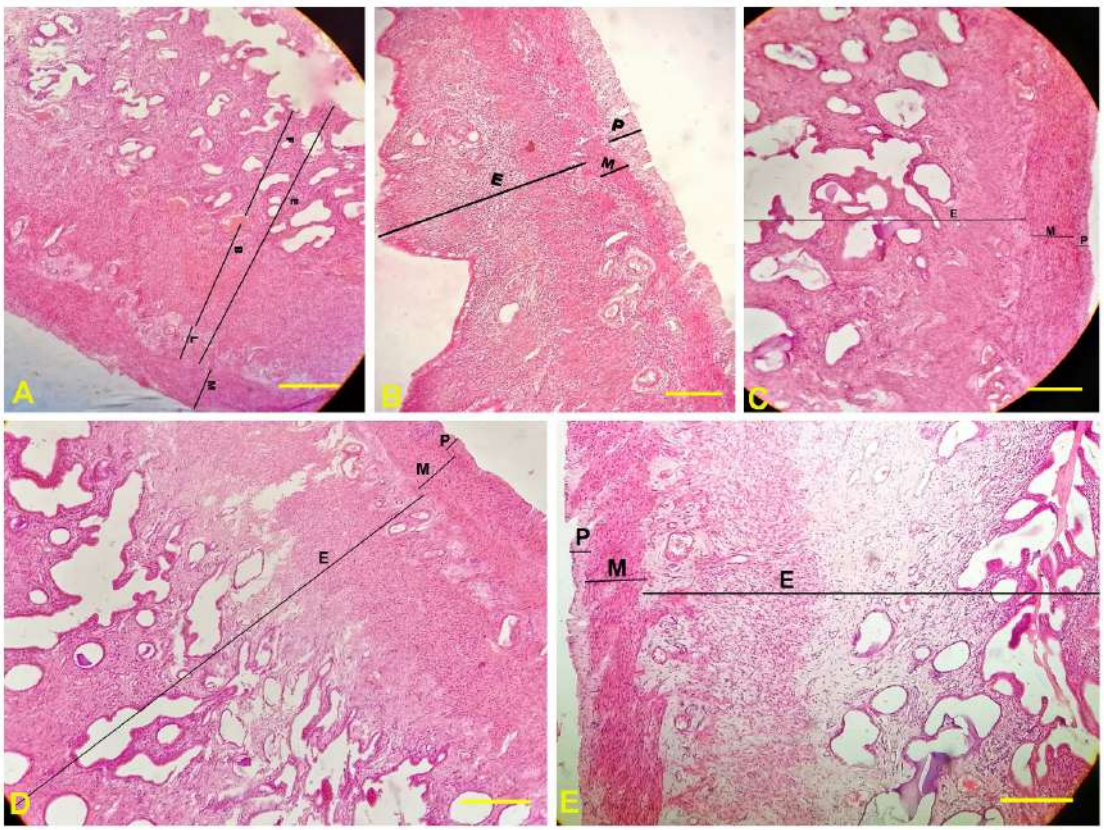

Figure 1. Cutting part of the uterine wall in the experimental groups

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Hematoxylin-Eosin Staining, 40X Magnification; Scale Bars: 100 µm.

Abbreviations: E, endometrium; M, myometrium; P, perimetrium; F, functional layer; B, basal layer; L, parenchyma.

Notes: A) In the control group, different layers of endometrium and myometrium are naturally seen.

B) The sham group, in which the number of glands is less and the parenchymal layer is almost absent; however, the epithelial layer is intact and its cylindrical cover is seen.

C) The endometrial layer in the testosterone group has tissue dispersion and its underlying material shows a large reduction, and the structure of the glands is destroyed.

D) In the chicory group, the endometrial layer, the number of its glands, the basal layer, and the connective tissue below it shows a normal state.

E) Tissue layers of the endometrium and uterine myometrium in the propolis group show normal state.

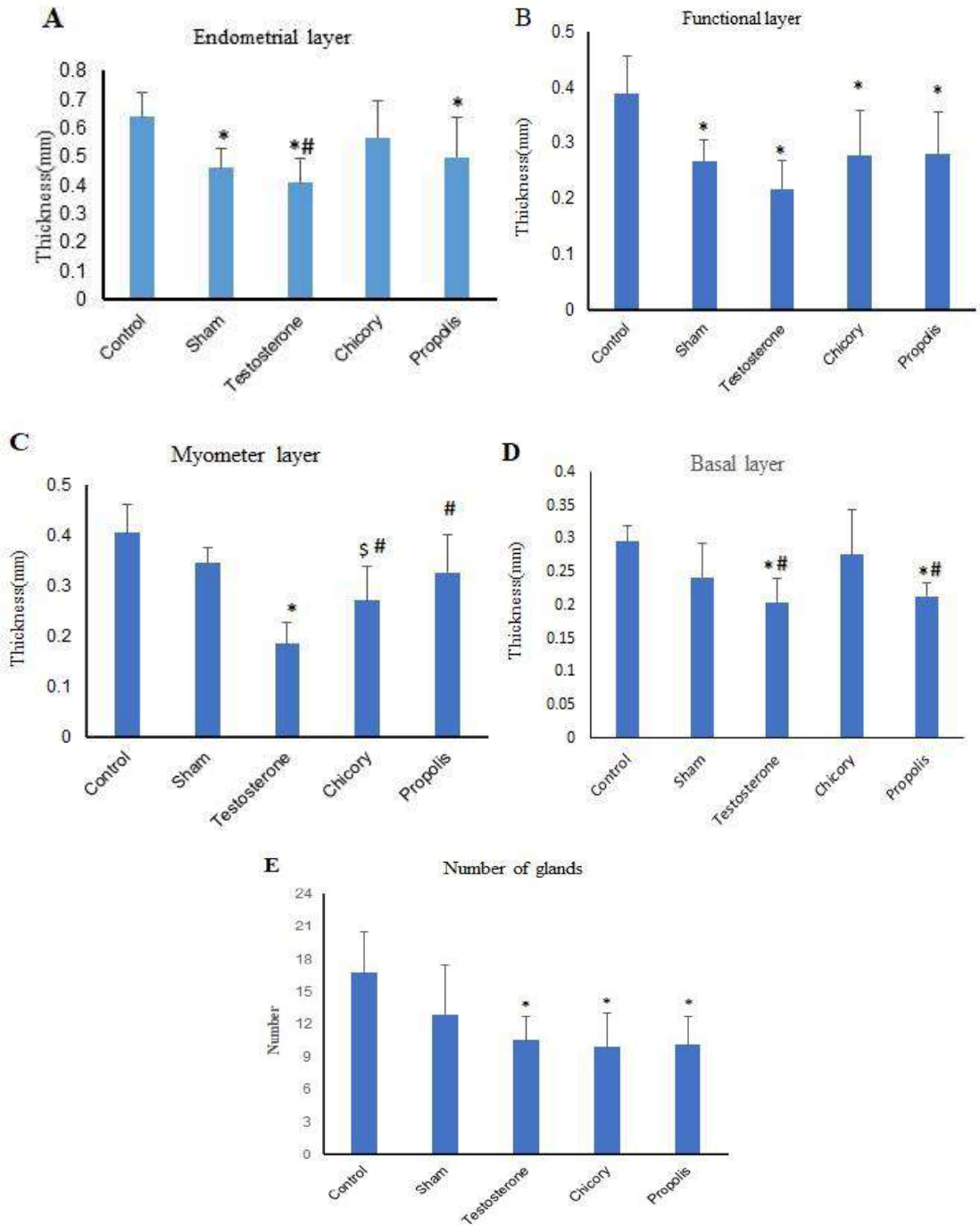

Figure 2. Changes in the different layers of the uterine tissue in the experimental groups

A) The thickness of the endometrial layer, B) the thickness of functional layer, C) the thickness of basal layer, D) the thickness of the myometrial layer, and E) the number of glands.

The following measures are according to the 1-way analysis of variance, the Tukey test (Mean±Standard Deviation):

*sign of a significant difference with the control group ($P<0.05$). # Sign of a significant difference between the testosterone and the chicory group ($P<0.05$). $ Sign of a significant difference between the chicory and the sham group ($P<0.05$).

Control: placebo; Sham: resistance training; Testosterone: resistance training+testosterone; Chicory: resistance training+testosterone+chicory; Propolis: resistance training+testosterone+Propolis.

Discussion

This study showed that 8 weeks of consuming propolis, chicory, and resistance training affected the body weight, uterus, estrus cycle, histopathology, and morphometry of the uterus of female mice that consumed testosterone enanthate. The results demonstrated that the body weight of the mice in the sham, testosterone, chicory, and propolis groups was increased compared to the control group, even though the body weight of the sham, chicory, and propolis mice was lower than that of the testosterone group; this amount was not significant. Studies by Saddick (2018), Santos et al. (2021), and Belardin et al. (2014) have also shown an increase in the weight of mice consuming AAS [26-28]. AAS is often prescribed at supra-physiological doses from 3 mg/kg body weight to 25 mg/kg body weight to increase muscle mass [29]. In the present study, the increase in the body weight in the testosterone enanthate groups may be due to the anabolic function of the drug. AAS binds to androgen receptors and causes hypertrophy of muscle fibers, reducing protein degradation and increasing nitrogen fixation [29].

Also, the uterine weight of the mice in the testosterone, chicory, and propolis groups was increased compared to the control group. In 2018, Saddick reported that uterine weight was a function of a pattern similar to that of mice during the AAS period, as the weight was increased during the period of consumption and decreased after withdrawal [26]. Other studies have also reported uterine weight gain [28, 30]. De Almida et al. (2011) stated that the changes in uterine weight are because of hormonal imbalances [31]. Uterine weight gain in the testosterone, chicory and propolis groups was probably because of the increased urethral secretion. The results from the study also demonstrated that the diestrus stage was increased in the testosterone, chicory, and propolis groups compared to the control group. However, the chicory and propolis groups were improving compared to the testosterone group. Elevated androgens may suppress the negative feedback loop, thereby reducing gonadotropin release and suppressing estrogen secretion in substances, and creating persistent diestrus [32]. Belardin et al. (2014) and Santos et al. (2021) also reported interference in the estrus cycle of female mice in addition to the continuation of the diestrus cycle caused by AAS [27, 28].

In the testosterone group, the superficial part of the endometrium lost its tissue structure, and its secretory glands were greatly reduced. In the deep parts, its structural shape showed considerable disarray, and its connection with the muscle layer was severed because of the problem of connective structures in some places. The myometrial layer in the testosterone group was thinner compared to the control group. Saddick (2018) reported that the effect of nandrolone decanoate on the ovaries and uterine tissue increases the myometrial layer and decreases the endometrial layer [26].

De Almida et al. (2011) reported that the uterine layers fully respond to AAS, leading to histological and morphological changes in endometrial atrophy. They also play an important role in this suppression by causing apparent disturbances in the physiology of reproduction [31]. Consistent with the mentioned studies, the present research also showed that the use of AAS causes histological and morphological changes in the uterine tissue. By examining the effects of testosterone enanthate along with resistance training, the present study showed that the thickness of the myometrial layer was decreased. The results from studies are different because of the type of steroid used, the dosage, and the duration of use.

On the other hand, it seems logical to assume that during intense exercise, the blood is preferentially transferred to skeletal muscle, and only a small amount is transferred to the visceral smooth muscle [33]. Intense exercise disrupts endocrine homeostasis, stimulates metabolic changes, and activates regulatory responses to induce new homeostasis. The female reproductive system is very sensitive to physiological stress and may change depending on the type, intensity, and duration of exercise [34]. Therefore, our findings are supported by intense exercise.

For this reason, the sham group also showed a decrease in the endometrial layer and the functional layer. The changes seen in the uterine tissue may be related to the effects of drug-induced protestation, as the steroid's ability to produce progestin-like molecules for metabolism with persistent diestrus may have contributed to the endometrial disorders associated with increased urethral secretion. Thus, the loss of morphological integrity of the myometrium in steroid-treated animals is significant and may be due to the expression of androgen receptor function in the layer. According to the researchers, androgen receptors in the myometrium may significantly affect the cells of the peripheral layer by the paracrine method, and studies have reported that the effect of post-consumption recovery periods depends on the dosage and time [28]. Another mechanism that points to histological and morphological changes in endometrial atrophy is that the biotransformation of AAS may affect the uterine tissue and the estrous cycle by altering aromatase activity, as a part of the AAS was transformed to

estradiol by the enzyme aromatase, which is important in altering the function of gonads. This process eventually leads to the conversion of estradiol to estrone and dihydroxy estrone [31].

Regarding the therapeutic effects of propolis and chicory scavengers on uterine damage caused by steroid use, the results of the present study showed that, compared to the testosterone, chicory and propolis groups could prevent the process of damage caused by testosterone enanthate. Propolis with more effects on the myometrium layer, and chicory with more effect on the endometrial layer further showed this treatment process. They had a significant difference compared to the control group. In women-related diseases, including uterine fibroids [35], chronic vaginitis, and ovarian toxicity, propolis has been used for treatment [36]. Also, it has been shown that the administration of aluminum sulfate and propolis has been a successful treatment to improve uterine leiomyoma [35]. Ali et al. (2018) used a randomized controlled clinical trial to demonstrate the beneficial effects of propolis on uterine leiomyoma. They concluded that the reduction in uterine fibroid volume in the propolis group was because of its direct effects on estrogen production in fibroids via autocrine-paracrine and P450 aromatase blockade [15]. The estrogenic effects of propolis have been attributed to the well-known phytoestrogen quercetin, camphor, and caffeine phenyl acid. Phytoestrogens have mixed estrogen agonist/antagonist properties that are clinically useful [37]. Propolis binds to estrogen receptors and causes estrogenic activity in estrogen receptors-expressing organs in the body; therefore, the main mechanism of action of propolis depends on its components [14]. Caffeine phenyl acid ester has been reported to be one of the most potent bioactive substances in propolis, which exerts its antitumor effect by inhibiting DNA synthesis [38, 39].

Regarding the scavenger effects of chicory, there was no study to show the use of chicory to treat steroids-caused effects in the uterine tissue. Some studies have shown that AAS users have used chicory to reduce its effects on the liver, kidneys, and brain; however, the healing properties of chicory have not been studied in the uterine tissue. Studies on the effects of chicory fiber on the reproductive function of pregnant mice have reported that chicory fiber maintains intestinal health by increasing intestinal function. It also improves reproductive function by changing the composition of intestinal microbiota [40]. According to the reports, the positive effects of chicory were more pronounced in male mice than in female mice. In female mice, chicory can regulate the peptidase gene. This gene plays an essential role in regulating innate and adaptive immunity [41]. Also, given the therapeutic properties of chicory, it has been reported that chicory can have an anti-inflammatory effect by severely inhibiting the induction of cyclooxygenase 2 by TNF-α and direct inhibition of cyclooxygenase enzymatic activities [42]. Therefore, according to studies, compared to chicory, it seems that propolis has a better-known position in treating diseases related to women. Therefore, this study is the first to deal with the treatment of the side effects of steroid use in utero through taking propolis and chicory scavengers. Thus, further research is needed for detailed comments on this issue.

The main limitation of the present study was the racial differences that may create differences in signal pathways between humans and laboratory animals that complicate the generalization of animal research results. It is suggested that the same study be performed on a human sample.

Conclusions

This study showed that the use of testosterone enanthate causes changes and damage in the structure and thickness of the uterine wall. In contrast, the use of propolis and chicory scavengers in female mice that consumed testosterone enanthate improved the side effects of consuming this type of steroid. Propolis had the greatest impact on the myometrium layer and chicory had the greatest impact on the endometrial layer in the healing process of the uterine. Therefore, propolis and chicory scavengers can be promising for the health of the female reproductive system and fertility.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article.

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

Investigation, writing-original draft, and approval of the final draft: All authors; methodology, supervision: Mohsen Akbarpour Beni and Ebrahim Cheraghi; Data analysis and cowriting the paper: Sodabeh Movahed and Mohsen Akbarpour Ben.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This article is the result of a dissertation on exercise physiology. The authors would like to thank the Research Affairs of the University of Qom.

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